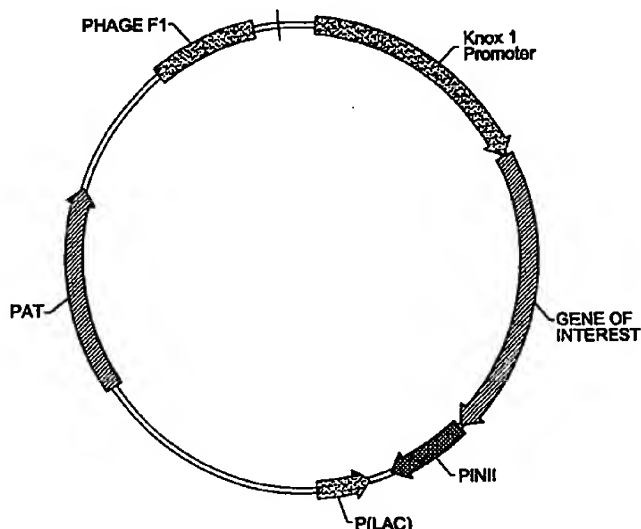




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(54) Title: ROOT-PREFERRED PROMOTERS AND THEIR USE



(57) Abstract

The present invention provides compositions and methods for regulating expression of heterologous nucleotide sequences in a plant. Compositions include a novel nucleotide sequence for a root-preferred promoter for the gene encoding *Knox1*, a *Knl*-like homeobox gene and sequences isolated therefrom. A method for expressing a heterologous nucleotide sequence in a plant using the promoter sequences disclosed herein is provided. The method comprises stably incorporating into the genome of a plant cell a nucleotide sequence operably linked to the root-preferred promoter of the present invention and regenerating a stably transformed plant that expresses the nucleotide sequence.

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ROOT-PREFERRED PROMOTERS AND THEIR USE

FIELD OF THE INVENTION

The present invention relates to the field of plant molecular biology, more particularly to regulation of gene expression in plants.

BACKGROUND OF THE INVENTION

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Expression of heterologous DNA sequences in a plant host is dependent upon the presence of an operably linked promoter that is functional within the plant host. Choice of the promoter sequence will determine when and where within the organism the heterologous DNA sequence is expressed. Where expression in
10 specific tissues or organs is desired, tissue-preferred promoters may be used. Where gene expression in response to a stimulus is desired, inducible promoters are the regulatory element of choice. In contrast, where continuous expression is desired throughout the cells of a plant, constitutive promoters are utilized. Additional regulatory sequences upstream and/or downstream from the core
15 promoter sequence may be included in expression constructs of transformation vectors to bring about varying levels of expression of heterologous nucleotide sequences in a transgenic plant.

Frequently it is desirable to express a DNA sequence in particular tissues or organs of a plant. For example, increased resistance of a plant to infection by soil-
20 and air-borne pathogens might be accomplished by genetic manipulation of the plant's genome to comprise a tissue-preferred promoter operably linked to a heterologous pathogen-resistance gene such that pathogen-resistance proteins are produced in the desired plant tissue.

Alternatively, it might be desirable to inhibit expression of a native DNA
25 sequence within a plant's tissues to achieve a desired phenotype. In this case, such inhibition might be accomplished with transformation of the plant to comprise a tissue-preferred promoter operably linked to an antisense nucleotide sequence,

such that expression of the antisense sequence produces an RNA transcript that interferes with translation of the mRNA of the native DNA sequence.

Thus far, the regulation of gene expression in plant roots has not been adequately studied despite the root's importance to plant development. To some degree this is attributable to a lack of readily available, root-specific biochemical functions whose genes may be cloned, studied, and manipulated. Genetically altering plants through the use of genetic engineering techniques and thus producing a plant with useful traits requires the availability of a variety of promoters. An accumulation of promoters would enable the investigator to design recombinant DNA molecules that are capable of being expressed at desired levels and cellular locales. Therefore, a collection of tissue-preferred promoters would allow for a new trait to be expressed in the desired tissue.

Thus, isolation and characterization of tissue-preferred, particularly root-preferred, promoters that can serve as regulatory regions for expression of heterologous nucleotide sequences of interest in a tissue-preferred manner are needed for genetic manipulation of plants.

SUMMARY OF THE INVENTION

Compositions and methods for regulating expression of heterologous nucleotide sequences in a plant are provided. Compositions comprise novel promoter sequences that initiate transcription in a root-preferred manner. More particularly, a transcriptional initiation region isolated from the plant gene *Knox1* is provided. Further compositions of the invention comprise the nucleotide sequence set forth in SEQ ID NO:1, a fragment of the nucleotide sequence set forth in SEQ ID NO:1 comprising at least 20 nucleotides, and the nucleotide sequence deposited as Patent Deposit No. 98917. The compositions of the invention further comprise nucleotide sequences having at least 70% identity to the sequence set forth in SEQ ID NO:1 or a fragment thereof, and nucleotide sequences that hybridized under stringent conditions to any one of the above mentioned sequences.

Compositions of the present invention also include an expression cassette

comprising a promoter, operably linked to a nucleotide sequence of interest, wherein said promoter is capable of driving expression of said nucleotide sequence in a plant cell and said promoter comprises the nucleotide sequences of the present invention. The invention further provides an expression vector comprising the
5 above mentioned expression cassette.

Compositions further include a plant or a plant cell having stably incorporated into their genomes an expression cassette comprising a promoter operably linked to a nucleotide sequence, wherein said promoter is capable driving expression of said nucleotide sequence in a plant cell and said promoter comprises
10 a nucleotide sequence of the present invention. Additionally, compositions include the seed of such plants.

Methods of the invention comprise a means to express a nucleotide sequence in a plant, said method comprising, stably incorporating into the genome of a plant cell an expression cassette comprising a promoter operably linked to a
15 nucleotide sequence, wherein said promoter is capable of initiating transcription of said nucleotide sequence in said plant cell and said promoter comprises a nucleotide sequence of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 schematically illustrates the plasmid vector P10322 comprising the GUS gene operably linked to the *Knox1* promoter.

Figure 2 shows transient expression levels of a *Knox1*::GUS DNA construct in root and shoot tissue from maize.

25 Figure 3 schematically illustrates the plasmid vector comprising a gene of interest operably linked to a promoter of the invention.

Figure 4 shows that the *knox1* gene is expressed in a high yielding hybrid maize strain in a density-response fashion.

DETAILED DESCRIPTION OF THE INVENTION

The compositions of the present invention comprise novel nucleotide sequences for plant promoters, particularly a "root-preferred" promoter for the *Knox1* gene, a *Kn1*-like homeobox gene, more particularly, the maize *Knox1* promoter. In particular, the present invention provides for an isolated nucleic acid molecule comprising the nucleotide sequences set forth in SEQ ID NO:1, and DNA sequences deposited in a bacterial host as Patent Deposit No. 98917, on October 7, 1998, and fragments and variants thereof.

10 A plasmid containing the nucleotide sequences of the invention were deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, and assigned Patent Deposit No. 98917. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112.

The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is

recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

5 The *Knox1* gene encodes a homeodomain protein that involved in the transcriptional control of developmentally regulated genes. Homeodomain proteins contain conserved regions known as "homeobox domains" consisting of a helix-turn-helix motif that has a DNA binding function. The maize *Knox1* gene is preferentially expressed in maize root tissue, although low levels of expression may occur in other tissues as well. See, for example, Vollbrecht *et al.* (1991) 10 *Nature* 350:241-243 and Kerstetter *et al.* (1994) *The Plant Cell* 6:1877-1887; herein incorporated by reference.

The *knox1* promoter sequences of the present invention direct expression of operably linked nucleotide sequences in a root-preferred manner. Therefore, the *knox1* promoter sequences find use in the root-preferred expression of an operably 15 linked nucleotide sequence of interest.

Furthermore, the level of *knox1* directed expression in the root is increased in high yielding maize hybrids planted at high density. Hence, the *knox1* promoter sequences find use in improving plant growth and/or crop yields under higher planting densities by regulating the expression of genes which improve the plant's 20 response to stresses induced under high density growth conditions. By "high density growth conditions" or "high density" is intended increasing the number of plants per area by about 50%, 100%, 200%, 300%, 400%, 600%, 800% or greater compared to the optimal growth density conditions of the plant. Alternatively, "high density" encompasses an increase in the number of plants per area from 25 about 50% to about 200%, about 200% to about 400%, about 400% to about 600%, or about 600% to about 800% or greater compared to the optimal growth density conditions of the plant.

Fragments and variants of the disclosed nucleotide sequence are also encompassed by the present invention. By "fragment" is intended a portion of the 30 nucleotide sequence. Fragments of a nucleotide sequence may retain biological activity and hence encompass fragments capable of driving root-preferred expression of an operably linked nucleotide sequence. Biologically active

fragments of the *knox1* promoter can also retain the ability to increase transcription of operably linked nucleotide sequences under high-density growth conditions.

Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not retain biological activity. Thus, fragments of a nucleotide
5 sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence of the invention.

Thus, a fragment of a *knox1* promoter nucleotide sequence may encode a biologically active portion of the *knox1* promoter or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A
10 biologically active portion of a *knox1* promoter can be prepared by isolating a portion of one of the *knox1* promoter nucleotide sequences of the invention, and assessing the activity of that portion of the *knox1* promoter. Nucleic acid molecules that are fragments of a *knox1* promoter nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650,
15 700, 800, 900, 1,000, 1,100, 1,200, 1,300, or 1,400, 1,600, 1,800, 2,000, 2,200, 2,500, 3,000, 3,200, 3,500, 4,000, 4,300 nucleotides, or up to the number of nucleotides present in a full-length *knox1* promoter nucleotide sequence disclosed herein (for example, 4315 nucleotides for SEQ ID NO:1).

By "variants" is intended substantially similar sequences. For nucleotide
20 sequences naturally occurring variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis. Generally,
25 variants of a particular nucleotide sequence of the invention will have at least 40%, 50%, 60%, 70%, generally at least 75%, 80%, 85%, preferably about 90% to 95% or more, and more preferably about 98% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters. Biologically active variants
30 are also encompassed by the present invention. Biologically active variants include, for example, the native promoter sequence of the invention having one or more nucleotide substitutions, deletions or insertions. Promoter activity may be

measured by using techniques such as Northern blot analysis, reporter activity measurements taken from transcriptional fusions, and the like. See, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), herein

5 incorporated by reference.

Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology*
10 (MacMillan Publishing Company, New York) and the references cited therein.

Variant nucleotide sequences also encompass sequences derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different *knox1* promoter sequences can be manipulated to create a new *knox1* promoter possessing the desired properties. In this manner,
15 libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994)
20 *Nature* 370:389-391; Cramer *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Cramer *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The nucleotide sequences of the invention can be used to isolate
25 corresponding sequences from other organisms, particularly other plants. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequence set forth herein. Sequences isolated based on their sequence identity to the entire *knox1* promoter sequence set forth herein or to fragments thereof are encompassed by the present
30 invention.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA

extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A*
5 *Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers,
10 vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The
15 hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ³²P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the *knox1* promoter sequence of the invention. Methods for preparation of probes for
20 hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire *knox1* sequence disclosed herein, or one or more
25 portions thereof, may be used as a probe capable of specifically hybridizing to corresponding *knox1* promoter sequences. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among *knox1* promoter sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes
30 may be used to amplify corresponding *knox1* promoter sequences from a chosen plant by PCR. This technique may be used to isolate additional coding sequences from a desired plant or as a diagnostic assay to determine the presence of coding

sequences in a plant. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

5 Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in
10 different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than
15 about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g.,
20 greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary
25 moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the
30 critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log$

M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Thus, isolated sequences that have promoter activity and which hybridize under stringent conditions to the *knox1* promoter sequence disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least 40% to 50% homologous, about 60% to 70% homologous, and even

about 75%, 80%, 85%, 90%, 95% to 98% homologous or more with the disclosed sequence. That is, the sequence identity of sequences may range, sharing at least 40% to 50%, about 60% to 70%, and even about 75%, 80%, 85%, 90%, 95% to 98% or more sequence identity.

5 The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

10 (a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

15 (b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid
20 a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

 Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two
25 sequences can be accomplished using a mathematical algorithm. Preferred, non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-method of
30 Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90; Huang *et al.* (1992) *CABIOS* 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See <http://www.ncbi.nlm.nih.gov>. Alignment may also be performed manually by inspection.

For purposes of the present invention, comparison of nucleotide or protein sequences for determination of percent sequence identity to the *knox1* promoter

sequences disclosed herein is preferably made using the Blast program (Version 2.0 or later) with its default parameters any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue

occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

- 5 (e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that
- 10 these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and
- 15 most preferably at least 95%.

- Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However,
- 20 stringent conditions encompass temperatures in the range of about 1°C to about 20°C lower than the T_m , depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is
- 25 created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

- (e)(ii) The term "substantial identity" in the context of a peptide indicates
- 30 that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified

comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

10 The coding sequence expressed by the promoters of the invention may be used to vary the phenotype of a plant. Various changes in phenotype are of interest including modifying expression of a gene in a plant root, altering a plant's pathogen or insect defense mechanism, increasing the plant's tolerance to herbicides, altering root development to respond to environmental stress, and the like. These results can be achieved by providing expression of heterologous or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes, transporters, or cofactors, or affecting nutrient uptake in the plant. These changes result in a change in phenotype of the transformed plant.

20 General categories of genes of interest for the present invention include, for example, those genes involved in information, such as Zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, and herbicide resistance. It is recognized that any gene of interest can be operably linked to the promoter of the invention and expressed in plant roots.

30 Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example *Bacillus thuringiensis* toxic protein genes (U.S. Patent Nos. 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593,881; Geiser *et al.* (1986) *Gene* 48:109); lectins (Van Damme *et al.* (1994) *Plant Mol. Biol.* 24:825); and the like.

Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (U.S. Patent Application No. 08/484,815, filed June 7, 1995); avirulence (avr) and disease resistance (R) genes (Jones *et al.* (1994) *Science* 266:789; Martin *et al.* (1993) *Science* 262:1432; Mindrinos *et al.* (1994) *Cell* 5 78:1089); and the like.

Herbicide resistance traits may be introduced into plants by genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (*e.g.*, the acetolactate synthase (ALS) gene containing mutations leading to such resistance, in particular 10 the S4 and/or Hra mutations), genes coding for resistance to herbicides that act to inhibit action of glutamine synthase, such as phosphinothricin or basta (*e.g.*, the *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS gene encodes resistance to the herbicide 15 chlorsulfuron.

Exogenous products include plant enzymes and products as well as those from other sources including prokaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like.

Examples of other applicable genes and their associated phenotype include 20 the gene which encodes viral coat protein and/or RNA, or other viral or plant genes that confer viral resistance; genes that confer fungal resistance; genes that promote yield improvement; and genes that provide for resistance to stress, such as dehydration resulting from heat and salinity, toxic metal or trace elements, or the like.

25 In other embodiments of the present invention, the *knox1* promoter sequences are operably linked to genes of interest that improve plant growth or increase crop yields under high plant density conditions. For example, the *knox1* promoter may be operably linked to nucleotide sequences expressing agronomically important genes that result in improved primary or lateral root 30 systems. Such genes include, but are not limited to, nutrient/water transporters and growth inducers. Examples of such genes, include but are not limited to, maize plasma membrane H⁺-ATPase (MHA2) (Frias *et al.* (1996) *Plant Cell* 8:1533-44);

AKT1, a component of the potassium uptake apparatus in Arabidopsis, (Spalding *et al.* (1999) *J Gen Physiol* 113:909-18); RML genes which activate cell division cycle in the root apical cells (Cheng *et al.* (1995) *Plant Physiol* 108:881); maize glutamine synthetase genes; (Sukanya *et al.* (1994) *Plant Mol Biol* 26:1935-46) and hemoglobin (Duff *et al.* (1997) *J. Biol. Chem* 27:16749-16752; Arredondo-Peter *et al.* (1997) *Plant Physiol.* 115:1259-1266; Arredondo-Peter *et al.* (1997) *Plant Physiol* 114:493-500 and references cited therein). The *knox1* promoter sequence may also be useful in expressing antisense nucleotide sequences of genes that negatively affects root development under high planting density conditions.

The heterologous nucleotide sequence operably linked to the promoter disclosed herein may be an antisense sequence for a targeted gene. By "antisense DNA nucleotide sequence" is intended a sequence that is in inverse orientation to the 5'-to-3' normal orientation of that nucleotide sequence. When delivered into a plant cell, expression of the antisense DNA sequence prevents normal expression of the DNA nucleotide sequence for the targeted gene. The antisense nucleotide sequence encodes an RNA transcript that is complementary to and capable of hybridizing to the endogenous messenger RNA (mRNA) produced by transcription of the DNA nucleotide sequence for the targeted gene. In this case, production of the native protein encoded by the targeted gene is inhibited to achieve a desired phenotypic response. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisense sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used. Thus, the promoter sequences disclosed herein may be operably linked to antisense DNA sequences to reduce or inhibit expression of a native protein in the plant root.

By "promoter" or "transcriptional initiation region" is intended a regulatory region of DNA usually comprising a TATA box capable of directing RNA

polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. A promoter may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, referred to as upstream promoter elements, which influence the transcription initiation rate. It is recognized that having identified the nucleotide sequences for the promoter regions disclosed herein, it is within the state of the art to isolate and identify further regulatory elements in the 5' untranslated region upstream from the particular promoter regions identified herein. Additionally, translational fusions may be provided. Such fusions include portions of the amino acid sequence. Thus the promoter regions disclosed herein are generally further defined by comprising upstream regulatory elements such as, those responsible for tissue and temporal expression of the coding sequence, enhancers and the like. In the same manner, the promoter elements, which enable expression in the desired tissue such as the root, can be identified isolated and used with other core promoters to confer root-preferred expression.

The regulatory sequences of the present invention, when operably linked to a heterologous nucleotide sequence of interest and stably incorporated into the plant genome drive "root-preferred" expression of the heterologous nucleotide sequence. By "root-preferred" is intended that expression of the heterologous nucleotide sequence is most abundant in the root. By root is intended any part of the root structure, including but not limited to, the root cap, apical meristem, protoderm, ground meristem, procambium, endodermis, cortex, vascular cortex, epidermis, and the like. While some level of expression of the heterologous nucleotide sequence may occur in other plant tissue types, expression occurs most abundantly in the root including primary, lateral, and adventitious roots.

By "heterologous nucleotide sequence" is intended a sequence that is not naturally occurring with the promoter sequence. While this nucleotide sequence is heterologous to the promoter sequence, it may be homologous, or native, or heterologous, or foreign, to the plant host.

The isolated promoter sequences of the present invention can be modified to provide for a range of expression levels of the heterologous nucleotide sequence. Thus, less than the entire promoter regions may be utilized and the ability to drive

expression of the coding sequence retained. However, it is recognized that expression levels of the mRNA may be decreased with deletions of portions of the promoter sequences. Generally, at least about 20 nucleotides of an isolated promoter sequence will be used to drive expression of a nucleotide sequence.

5 It is recognized that to increase transcription levels enhancers may be utilized in combination with the promoter regions of the invention. Enhancers are nucleotide sequences that act to increase the expression of a promoter region. Enhancers are known in the art and include the SV40 enhancer region, the 35S enhancer element, and the like.

10 Modifications of the isolated promoter sequences of the present invention can provide for a range of expression of the heterologous nucleotide sequence. Thus, they may be modified to be weak promoters or strong promoters. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended at levels of about 1/10,000
15 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a strong promoter drives expression of a coding sequence at a high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

 It is recognized that the promoters of the invention thereof may be used
20 with their native coding sequences to increase or decrease expression, thereby resulting in a change in phenotype of the transformed plant.

 The nucleotide sequences for the root-preferred promoters disclosed in the present invention, as well as variants and fragments thereof, are useful in the genetic manipulation of any plant when operably linked with a heterologous
25 nucleotide sequence whose expression is to be controlled to achieve a desired phenotypic response. By "operably linked" is intended the transcription of the heterologous nucleotide sequence is under the influence of the promoter sequence. By "operably linked" is also intended the joining of two nucleotide sequences such that the coding sequence of each DNA fragment remain in the proper reading
30 frame. In this manner, the nucleotide sequences for the promoters of the invention may be provided in expression cassettes along with heterologous nucleotide

sequences of interest for expression in the plant of interest, more particularly in the root of the plant.

Such expression cassettes will comprise a transcriptional initiation region comprising the promoter nucleotide sequences of the present invention, or variants
5 or fragments thereof, operably linked to a heterologous nucleotide sequence whose expression is to be controlled by the root-preferred promoter disclosed herein. Such an expression cassette is provided with a plurality of restriction sites for insertion of the nucleotide sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable
10 marker genes.

The expression cassette will include in the 5'-to-3' direction of transcription, a transcriptional and translational initiation region, a heterologous nucleotide sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the
15 transcriptional initiation region comprising the promoter nucleotide sequence of the present invention, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau *et al.* (1991) *Mol. Gen. Genet.*
20 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* 1989) *Nucleic Acids Res.* 17:7891-7903; Joshi heterologous *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

The expression cassette comprising the promoter sequence of the present
25 invention operably linked to a heterologous nucleotide sequence may also contain at least one additional nucleotide sequence for a gene to be cotransformed into the organism. Alternatively, the additional sequence(s) can be provided on another expression cassette.

Where appropriate, the heterologous nucleotide sequence whose expression
30 is to be under the control of the root-preferred promoter sequence of the present invention and any additional nucleotide sequence(s) may be optimized for increased expression in the transformed plant. That is, these nucleotide sequences

can be synthesized using plant preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred nucleotide sequences. See, for example, U.S. Patent Nos. 5,380,831 and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by
5 reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene
10 expression. The G-C content of the heterologous nucleotide sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in
15 the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Nat. Acad. Sci. USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986));
20 MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154:9-20); human immunoglobulin heavy-chain binding protein (BiP) (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) *Molecular Biology of RNA*, pages 237-
25 256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also Della-Cioppa *et al.* (1987) *Plant Physiology* 84:965-968. Other methods known to enhance translation and/or mRNA stability can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be
30 manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be

involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, for example, transitions and transversions, may be involved.

5 Reporter genes or selectable marker genes may be included in the expression cassettes. Examples of suitable reporter genes known in the art can be found in, for example, Jefferson *et al.* (1991) in *Plant Molecular Biology Manual*, ed. Gelvin *et al.* (Kluwer Academic Publishers), pp. 1-33; DeWet *et al.* (1987) *Mol. Cell. Biol.* 7:725-737; Goff *et al.* (1990) *EMBO J.* 9:2517-2522; Kain *et al.* 10 (1995) *BioTechniques* 19:650-655; and Chiu *et al.* (1996) *Current Biology* 6:325-330.

Selectable marker genes for selection of transformed cells or tissues can include genes that confer antibiotic resistance or resistance to herbicides. Examples of suitable selectable marker genes include, but are not limited to, genes 15 encoding resistance to chloramphenicol (Herrera Estrella *et al.* (1983) *EMBO J.* 2:987-992); methotrexate (Herrera Estrella *et al.* (1983) *Nature* 303:209-213; Meijer *et al.* (1991) *Plant Mol. Biol.* 16:807-820); hygromycin (Waldron *et al.* (1985) *Plant Mol. Biol.* 5:103-108; Zhijian *et al.* (1995) *Plant Science* 108:219-227); streptomycin (Jones *et al.* (1987) *Mol. Gen. Genet.* 210:86-91); 20 spectinomycin (Bretagne-Sagnard *et al.* (1996) *Transgenic Res.* 5:131-137); bleomycin (Hille *et al.* (1990) *Plant Mol. Biol.* 7:171-176); sulfonamide (Guerineau *et al.* (1990) *Plant Mol. Biol.* 15:127-136); bromoxynil (Stalker *et al.* (1988) *Science* 242:419-423); glyphosate (Shaw *et al.* (1986) *Science* 233:478-481); phosphinothricin (DeBlock *et al.* (1987) *EMBO J.* 6:2513-2518).

25 Other genes that could serve utility in the recovery of transgenic events but might not be required in the final product would include, but are not limited to, examples such as GUS (b-glucoronidase; Jefferson (1987) *Plant Mol. Biol. Rep.* 5:387), GFP (green florescence protein; Chalfie *et al.* (1994) *Science* 263:802), luciferase (Riggs *et al.* (1987) *Nucleic Acid Res.* 15(19):8115 and Luehrsen *et al.* 30 (1992) *Methods Enzymol.* 216:397-414) and the maize genes encoding for anthocyanin production (Ludwig *et al.* (1990) *Science* 247:449).

The expression cassette comprising the particular promoter sequence of the present invention operably linked to a nucleotide sequence of interest can be used to transform any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, root, and the like can be obtained.

- 5 Such plant species, including, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet
- 10 (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos*
- 15 *mucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*),
- 20 sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*).

- 25 Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example,
- 30 pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga*

canadensis); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), more preferably corn and soybean plants, yet more preferably corn plants.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Pat No. 5,563,055), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney *et al.*, U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag,

- Berlin) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De
- 5 Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports*
- 10 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

- The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986)
- 15 *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having root-preferred expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that root-preferred expression of the desired phenotypic characteristic is stably maintained and inherited and then
- 20 seeds harvested to ensure root-preferred expression of the desired phenotypic characteristic has been achieved.

The following examples are offered by way of illustration and not by way of limitation.

25

EXPERIMENTAL

The promoter region for the maize gene *knox1* was isolated from maize plants. The sequence for the *knox1* promoter is set forth in SEQ ID NO: 1. The method for its isolation is described below.

Example 1: Isolation of Promoter Sequences

One to three-week old *Zea mays* cv. B73 were grown in soil in the greenhouse. Whole root and whole leaf tissue were harvested and immediately
5 frozen in liquid nitrogen. Total RNA was harvested using TriPure Reagent (Boehringer Mannheim, Indianapolis, IN) and the manufacturer's protocol. PolyA RNA was isolated from 1-3 mg total RNA using a magnetic-bead-poly dT method from Promega (Madison, WI). Exactly 4.2 µg and 6 µg of root and leaf polyA RNA, respectively, were denatured with a solution of 50% formamide, 6%
10 formaldehyde, 0.5X MOPS and 0.01% Bromphenol Blue by heating the RNA mixtures at 65°C for 15 minutes then placed on ice. The RNA was loaded on a 1.2% SeaKEM GTG agarose gel with 1X MOPS and 2% Formaldehyde and run at 70 volts for 2 hours. Using 20X SSC, the RNA gels were transferred overnight to a Nytran membrane using the Turboblot System (GIBCO BRL, Gaithersburg,
15 MD). Following blotting, the membranes were air dried and crosslinked with UV using a Stratalinker at a setting of 1200 microjoules (Stratagene, LaJolla, CA). The membranes were then prehybridized with 10 ml of 1X "Expresshyb" solution (Clontech, Palo Alto, CA) for 1 hour at 65°C. The 5' and 3' *KNOX1* probes were radiolabeled using Redivue random priming labeling method from Amersham
20 (UK) with ³²P-α-dCTP and added to fresh 1X Expresshyb solution for hybridization to the membrane at 65°C overnight. The membranes were washed two times with 2X SSC, 0.1% SDS for 10 minutes at room temperature. This was followed by a single stringent wash with 0.1X SSC, 0.1% SDS for 30 minutes at 50°C. The membranes were exposed to Kodak XAR X-ray film at -80°C with
25 DuPont Intensifier screen for 4 days. Endogenous transcripts from the maize *knox1* gene were detected by Northern analysis. A strong *knox1* mRNA band was observed in root but not in leaf tissues (data not shown).

Example 2: Expression Data Using Promoter Sequences

Nine (9) μ g of PHP10322 (Figure 1) plus 1 μ g of Ubi::LUC to act as a standard control were precipitated onto tungsten particles and bombarded into 3-day old seedlings essentially as described in Tomes *et al.* Shoots and roots were harvested separately and measured for GUS activity using GUS Light Kit from Tropix (San Diego, CA) following the manufacturer's protocol. Protein assays were conducted with Bradford Protein Assay from BioRad (Hercules, CA) Emoryville kit. Results are shown in Figure 2. The data show normalized GUS units as recorded on a Luminometer to soluble protein.

Example 3: Transformation and Regeneration of Maize Callus

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the *knox1* promoter sequence operably linked to a nucleotide sequence of interest (Figure 3). The plasmid further contains the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37) that confers resistance to the herbicide Bialaphos. Transformation is performed as follows. All media recipes are in the Appendix.

Preparation of Target Tissue

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

The plasmid vector shown in figure 3 is precipitated onto 1.1 μ m (average diameter) tungsten pellets using a CaCl_2 precipitation procedure as follows:

100 µl prepared tungsten particles in water
10 µl (1 µg) DNA in TrisEDTA buffer (1 µg total)
100 µl 2.5 M CaCl₂
10 µl 0.1 M spermidine

5

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with
10 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

15

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

20

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection,
25 selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are
30 well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber,

subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for the an altered phenotypic trait.

5 Example 4. Expression of the *knox1* gene under high density growth conditions

Two maize hybrids, 3394 and 3306, were planted at high-density equivalents to 35,000 plant/acre. Half of each hybrid was thinned to an equivalent of 4,000 plants/acre. The 3394 hybrid yields very well at the high density relative
10 to the 3306 hybrid. Root RNA was isolated using standard protocols from two developmental stages, V8 and V12-R1. The RNA was converted to a probe and applied onto the Affymetrix Genechip supplied for Pioneer's corn EST database using published protocols. The GeneChip includes representative oligos for 1501 Pioneer EST clones. Each gene is represented by 20 oliogs of perfect matches and
15 20 oligos of single base pair mismatch. Using Affymetrix methods for measuring gene expression levels, we determined that the *knox1* gene is expressed in the high yielding hybrid in a density-response fashion. This suggests that the *knox1* promoters may harbor promoter elements that stimulate expression in the appropriate hybrids under the high-density growth conditions. Figure 4 depicts the
20 relative mRNA levels for two parts of *knox1* (5' and 3' end loaded on the GeneChip) and two controls, polyubiquitin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA levels were normalized to the 3306 hybrid at 4K density planting for each gene. The *knox1* gene was expressed 2-2.5 times higher in the 3394 hybrid at high planting densities that at low planting densities
25 whereas the controls were regulated at similar levels in roots.

APPENDIX272 V

Ingredient	Amount	Unit
D-I H ₂ O	950.000	ml
MS Salts (GIBCO 11117-074)	4.300	G
Myo-Inositol	0.100	G
MS Vitamins Stock Solution ##	5.000	ml
Sucrose	40.000	G
Bacto-Agar @	6.000	G

Directions:

- 5 @ = Add after bringing up to volume

Dissolve ingredients in polished D-I H₂O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

- 10 ## = Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occurs, then make fresh stock.
- 15 Total Volume (L) = 1.00

288 J

Ingredient	Amount	Unit
D-I H ₂ O	950.000	ml
MS Salts	4.300	G
Myo-Inositol	0.100	G
MS Vitamins Stock Solution ##	5.000	ml
Zeatin .5mg/ml	1.000	ml
Sucrose	60.000	G
Gelrite @	3.000	G
Indoleacetic Acid 0.5 mg/ml #	2.000	ml
0.1mM Absciscic Acid	1.000	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

- 5 Dissolve ingredients in polished D-I H₂O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

Add 3.5g/L of Gelrite for cell biology.

- 10 ## = Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occurs, then make fresh stock.
- 15 Total Volume (L) = 1.00

560 R

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	G
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	30.000	G
2, 4-D 0.5mg/ml	4.000	ml
Gelrite @	3.000	G
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H₂O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H₂O

Sterilize and cool to room temp.

10 Total Volume (L) = 1.00

560 Y

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	MI
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	G
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	MI
Thiamine.HCL 0.4mg/ml	1.250	MI
Sucrose	120.000	G
2,4-D 0.5mg/ml	2.000	MI
L-Proline	2.880	G
Gelrite @	2.000	G
Silver Nitrate 2mg/ml #	4.250	MI

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H₂O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H₂O

Sterilize and cool to room temp.

10 ** Autoclave less time because of increased sucrose**

Total Volume (L) = 1.00

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains.

All publications and patent applications are herein incorporated by reference to the
5 same extent as if each individual publication or patent application was specifically
and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way
of illustration and example for purposes of clarity of understanding, it will be
obvious that certain changes and modifications may be practiced within the scope
10 of the appended claims.

Applicant's or agent's file reference	5718-52-1	International application No.	PCT/US99/
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INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 2, line 27; page 4, lines 8 and 12; page 35, lines 9 and 26; page 36, line 14; page 37, lines 10 and 31	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depository institution American Type Culture Collection	
Address of depository institution (including postal code and country) 10801 University Blvd. Manassas, VA 20110-2209 US	
Date of deposit 07 October 1998	Accession Number 98917
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indicators are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only	
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For International Bureau use only	
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Authorized officer	

THAT WHICH IS CLAIMED:

1. An isolated promoter that is capable of driving expression in a plant cell, wherein said promoter comprises a nucleotide sequence selected from the group consisting of:
- 5 a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:1;
- b) a nucleotide sequence comprising the sequence deposited as Patent Deposit No. 98917;
- 10 c) a nucleotide sequence comprising a fragment of the sequence set forth in SEQ ID NO:1;
- d) a nucleotide sequence comprising at least 20 contiguous nucleotides of the sequence set forth in SEQ ID NO:1;
- e) a nucleotide sequence comprising a sequence having at least 15 70% identity to the sequence set forth in SEQ ID NO:1 or a fragments thereof;
- f) a sequence that hybridizes under stringent conditions to any one of a), b), c), d), or e) under stringent conditions.
2. An expression cassette comprising a promoter operably linked to a nucleotide sequence, wherein said promoter is capable of driving expression of said nucleotide sequence in a plant cell, wherein said promoter comprises a nucleotide sequence selected from the group consisting of:
- a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:1;
- 25 b) a nucleotide sequence comprising the sequence deposited as Patent Deposit No. 98917;
- c) a nucleotide sequence comprising a fragment of the sequence set forth in SEQ ID NO:1;
- d) a nucleotide sequence comprising at least 20 contiguous 30 nucleotides of the sequence set forth in SEQ ID NO:1;
- e) a nucleotide sequence comprising a sequence having at least 70% identity to the sequence set forth in SEQ ID NO:1 or a fragments thereof;

f) a sequence that hybridizes under stringent conditions to any one of a), b), c), d), or e) under stringent conditions.

3. An expression vector comprising the expression cassette of claim 2.

5

4. A plant having stably incorporated into its genome an expression cassette comprising a promoter operably linked to a nucleotide sequence, wherein said promoter is capable driving expression of said nucleotide sequence in a plant cell and said promoter comprises a nucleotide sequence selected from the group consisting of:

10

a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:1;

b) a nucleotide sequence comprising the sequence deposited as Patent Deposit No. 98917;

15

c) a nucleotide sequence comprising a fragment of the sequence set forth in SEQ ID NO:1;

d) a nucleotide sequence comprising at least 20 contiguous nucleotides of the sequence set forth in SEQ ID NO:1;

e) a nucleotide sequence comprising a sequence having at least 20 70% identity to the sequence set forth in SEQ ID NO:1 or a fragments thereof;

f) a sequence that hybridizes under stringent conditions to any one of a), b), c), d), or e) under stringent conditions.

5. The plant of claim 4, wherein said plant is a monocot.

25

6. The plant of claim 5, wherein said monocot is maize.

7. The plant of claim 4, wherein said plant is a dicot.

30

8. Seed of the plant of claim 4.

9. A method for expressing a nucleotide sequence in a plant, said method comprising, stably incorporating into the genome a plant cell an expression cassette comprising a promoter operably linked to a nucleotide sequence, wherein said promoter is capable of initiating transcription of said nucleotide sequence in said plant cell and said promoter comprises a nucleotide sequence selected from the group consisting of:
- a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:1;
 - b) a nucleotide sequence comprising the sequence deposited as Patent Deposit No. 98917;
 - c) a nucleotide sequence comprising a fragment of the sequence set forth in SEQ ID NO:1;
 - d) a nucleotide sequence comprising at least 20 contiguous nucleotides of the sequence set forth in SEQ ID NO:1;
 - e) a nucleotide sequence comprising a sequence having at least 70% identity to the sequence set forth in SEQ ID NO:1 or fragments thereof;
 - f) a sequence that hybridizes under stringent conditions to any one of a), b), c), d), or e) under stringent conditions.

10. The method of claim 9, wherein said nucleotide sequence is selectively expressed in the root.

11. A plant cell having stably incorporated into its genome an expression cassette comprising a promoter operably linked to a nucleotide sequence, wherein said promoter is capable of initiating transcription of said nucleotide sequence in a plant cell and said promoter comprises a nucleotide sequence selected from the group consisting of:

- a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:1;
- b) a nucleotide sequence comprising the sequence deposited as Patent Deposit No. 98917;

- c) a nucleotide sequence comprising a fragment of the sequence set forth in SEQ ID NO:1;
 - d) a nucleotide sequence comprising at least 20 contiguous nucleotides of the sequence set forth in SEQ ID NO:1;
 - 5 e) a nucleotide sequence comprising a sequence having at least 70% identity to the sequence set forth in SEQ ID NO:1 or fragments thereof;
 - f) a sequence that hybridizes under stringent conditions to any one of a), b), c), d), or e) under stringent conditions.
- 10 12. The plant cell of claim 11, wherein said plant cell is from a monocotyledonous plant.
13. The plant cell of claim 12, wherein said plant is maize.
- 15 14. The plant cell of claim 11, wherein said plant cell is from a dicotyledonous plant.

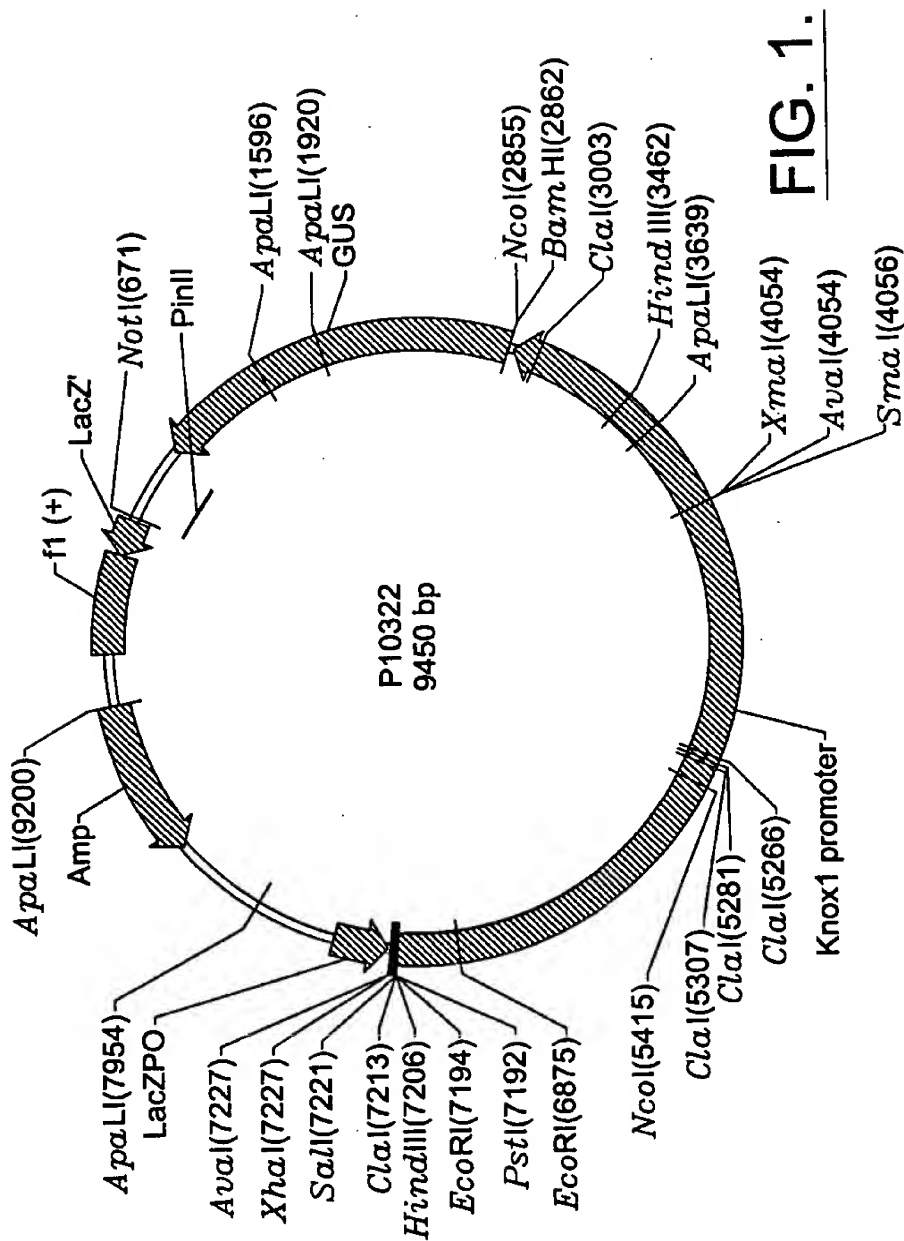
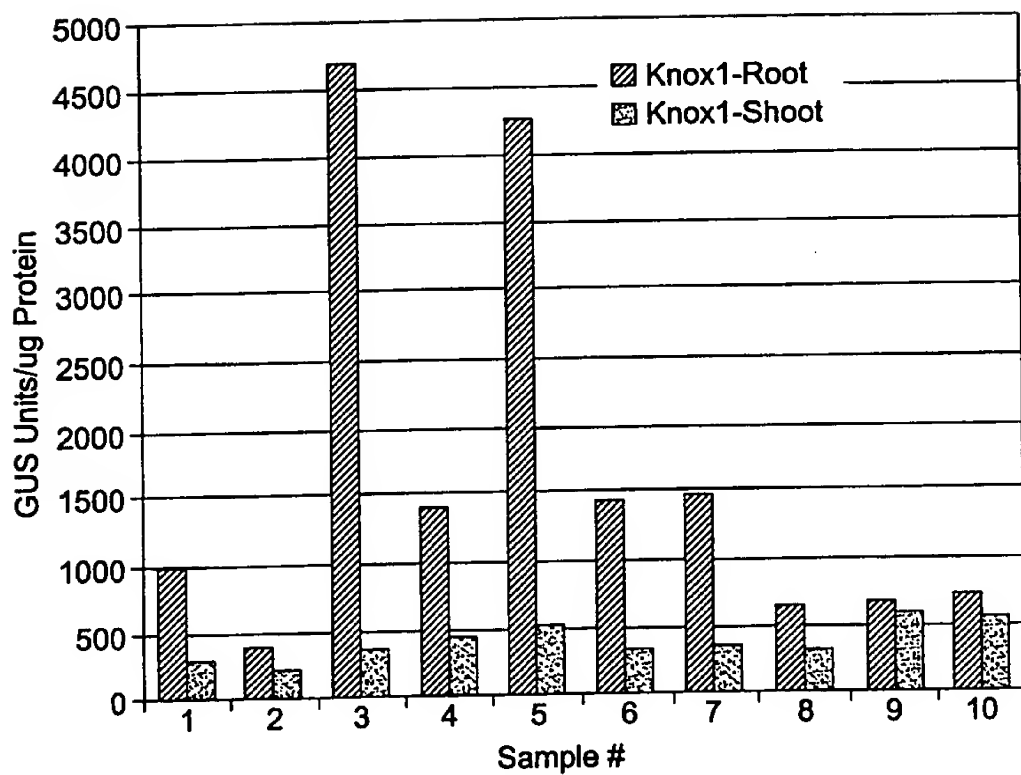
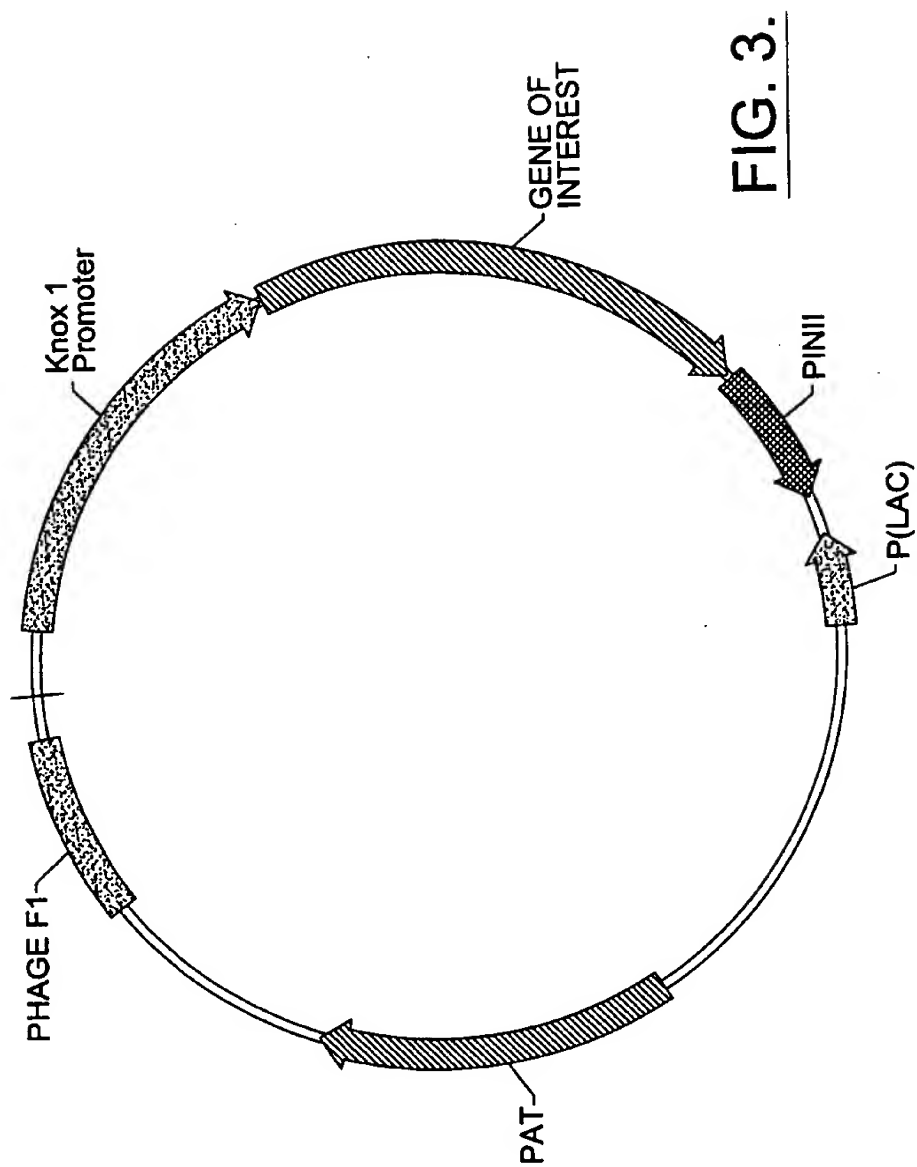


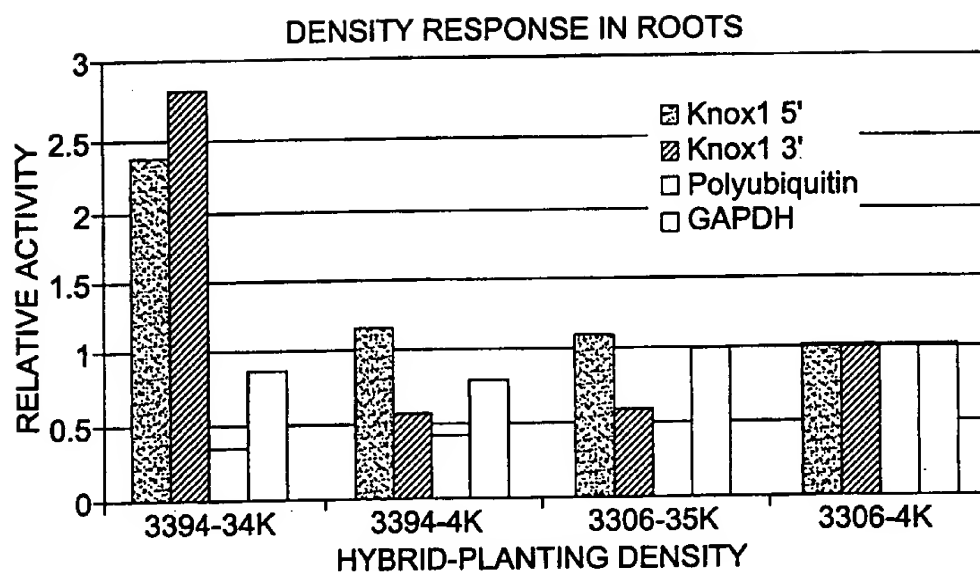
FIG. 1.

2/4

FIG. 2.



4/4

**FIG. 4.**

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Sims, Lynne

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/27236

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/10 C12N15/31 C12N15/63 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 04196 A (IMP CANCER RES TECH ;VILE RICHARD GEOFFREY (GB); HART IAN ROGER (G) 3 March 1994 (1994-03-03) page 27, line 18 SEQ ID NO:7 page 67	1-9, 11-14
X	WO 94 04562 A (GEN HOSPITAL CORP) 3 March 1994 (1994-03-03) figure 14	1-9, 11-14
A	WO 97 44448 A (PIONEER HI BRED INT) 27 November 1997 (1997-11-27) -/-	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Burkhardt, P

INTERNATIONAL SEARCH REPORT

Interr .al Application No

PCT/US 99/27236

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